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(54) Title: METHOD OF CANCER TREATMENT

(57) Abstract

Treatment of solid tumors, including their metastases, without radiation, surgery or standard chemotherapeutic agents. *Ex vivo* stimulation of cells and reinfusion for cancer therapy. Culture of host cells in culture media and re-infusion of host cells.

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METHOD OF CANCER TREATMENT

RELATED APPLICATION DATA

This application is a continuation-in-part application of copending Application Serial No. 07/891,718, filed June 1, 1992, which is a continuation-in-part application of Application Serial No. PCT/US91/00342, which is a continuation-in-part application of Application Serial No. 07/466,577, filed on January 17, 1990, which is a continuation-in-part application of Application Serial No. 07/416,530, filed Ocotber 3, 1989.

FIELD OF THE INVENTION

The invention generally relates to the treatment of cancer, and, more specifically, to the treatment of solid tumors, including their metastases, without radiation, surgery or standard chemotherapeutic agents.

BACKGROUND

Therapy for cancer has largely involved the use of radiation, surgery and chemotherapeutic agents. However, results with these measures, while beneficial in some tumors, has had only marginal or no effect in many others. Furthermore, these approaches have often unacceptable toxicity.

Both radiation and surgery suffer from the same theoretical drawback. It has been recognized that, given that a single clonogenic malignant cell can give rise to sufficient progeny to kill the host, the entire population of neoplastic cells must be eradicated. See generally, Goodman and Gilman The Pharmacological Basis of Therapeutics (Pergamon Press, 8th Edition) (pp. 1202-1204). This concept of "total cell kill" implies that total excision of a tumor is necessary for a surgical approach, and complete

destruction of all cancer cells is needed in a radiation approach, if one is to achieve a cure. In practice this is rarely possible; indeed, where there are metastases, it is impossible.

The term "chemotherapy" simply means the treatment of disease with chemical substances. The father of chemotherapy, Paul Ehrlich, imagined the perfect chemotherapeutic as a "magic bullet;" such a compound would kill invading organisms without harming the host. This target specificity is sought in all types of chemotherapeutics, including anticancer agents.

However, specificity has been the major problem with anticancer agents. In the case of anticancer agents, the drug needs to distinguish between host cells that are cancerous and host cells that are not cancerous. The vast bulk of anticancer drugs are indiscriminate at this level. Typically anticancer agents have negative hematological effects (e.g., cessation of mitosis and disintegration of formed elements in marrow and lymphoid tissues), and immunosuppressive action (e.g., depressed cell counts), as well as a severe impact on epithelial tissues (e.g., intestinal mucosa), reproductive tissues (e.g., impairment of spermatogenesis), and the nervous system. P. Calabresi and B.A. Chabner, In: Goodman and Gilman The Pharmacological Basis of Therapeutics (Pergamon Press, 8th Edition) (pp. 1209-1216).

Success with chemotherapeutics as anticancer agents has also been hampered by the phenomenon of multiple drug resistance, resistance to a wide range of structurally unrelated cytotoxic anticancer compounds. J.H. Gerlach *et al., Cancer Surveys*, 5:25-46 (1986). The underlying cause of progressive drug resistance may be due to a small population of drug-resistant cells within the tumor (e.g., mutant cells) at the time of diagnosis. J.H. Goldie and

Andrew J. Coldman, *Cancer Research*, 44:3643-3653 (1984). Treating such a tumor with a single drug first results in a remission, where the tumor shrinks in size as a result of the killing of the predominant drug-sensitive cells. With the drug-sensitive cells gone, the remaining drug-resistant cells continue to multiply and eventually dominate the cell population of the tumor.

Treatment at the outset with a combination of drugs was proposed as a solution, given the small probability that two or more different drug resistances would arise spontaneously in the same cell. V.T. DeVita, Jr., *Cancer*, 51:1209-1220 (1983). However, it is now known that drug resistance is due to a membrane transport protein, "P-glycoprotein," that can confer general drug resistance. M.M. Gottesman and I. Pastan, *Trends in Pharmacological Science*, 9:54-58 (1988). Phenotypically, the tumor cells show, over time, a reduced cellular accumulation of all drugs. In short, combination chemotherapy appears not to be the answer.

What is needed is a specific anticancer approach that is reliably tumoricidal to a wide variety of tumor types. Importantly, the treatment must be effective with minimal host toxicity.

SUMMARY OF THE INVENTION

The invention generally relates to the treatment of cancer, and, more specifically, to the treatment of solid tumors, including their metastases, without radiation, surgery or standard chemotherapeutic agents. In one embodiment, the invention involves using superantigens, including SEA and SEB, to stimulate tumor draining lymph node cells *ex vivo*, allowing them to differentiate into tumor specific immune effector cells. The cells are then reintroduced into the same host to mediate anticancer therapeutic effects. In another embodiment, the stimulated

cells are introduced into a different host. In still a third embodiment, the cells are established as a cell line for continuous anticancer use.

In one embodiment, lymphocytes are obtained early in life from cancer-free hosts. The cells are stored in appropriate containers under liquid nitrogen using conventional techniques (e.g., DMSO, culture media, fetal calf serum, etc.) until the onset of disease. At this point, the cells may be thawed, and cultured and stimulated in the manner of the present invention for reinfusion.

Alternatively, an established cell line may be made from cancer-free hosts. The cell line can be stored as above. On the other hand, they may be passed continuously in culture until use.

The *ex vivo* stimulation method has decided advantages over direct intravenous injection of superantigens, namely: 1) the superantigens are ensured of contacting their appropriate target cell, namely, T lymphocytes; in other words, stimulation is specific; 2) stimulation in culture allows for the removal of the stimulating antigens prior to reintroduction of the cells in the host, i.e., the host is exposed to only very small amounts of superantigens *in vivo*; and 3) lack of systemic exposure to the stimulating antigens precludes significant interference with naturally occurring or induced antibodies to superantigens.

The present invention demonstrates that superantigens can reliably produce tumoricidal reactions to a wide variety of tumor types. Moreover, success is achieved with minimal host toxicity using the *in vitro* sensitization technique.

In its simplest form, the present invention offers a method for inducing a tumoricidal reaction *in vivo* comprising contacting cells with superantigens *ex vivo* and infusing them into a tumor-bearing host. The cells are typically hematopoietic cells, such as peripheral blood

lymphocytes, spleen cells, tumor-infiltrating lymphocytes or lymph node cells. Where they are lymph node cells, it is preferred that they are from a tumor-bearing host. The superantigens may comprise enterotoxins of *Staphylococcus aureus*, or synthetic polypeptides with substantial structural homology and statistically significant sequence homology to natural superantigens.

The present invention offers a method of human cancer treatment comprising: a) providing a human cancer patient; b) obtaining hematopoietic cells from said patient; c) contacting said cells *ex vivo* with one or more superantigens to generate stimulated cells; and d) re-introducing said stimulated cells into said patient so as to induce an *in vivo* therapeutic, tumoricidal reaction. Preferably the hematopoietic cells are cultured in culture media containing enterotoxins and the cultured cells are washed prior to re-introducing said stimulated cells into said patient so as to essentially avoid introducing enterotoxins *in vivo*.

The culture cells can be viewed as a reagent for treating cancer, comprising T cells sensitized to a growing tumor and stimulated with superantigens. Preferably, the T cells are suspended in media suitable for intravenous administration to a human cancer patient, such as a media comprising a physiological buffered saline solution.

While not limited to any mechanism, it is believed that culturing the cells in the manner proposed results in subset enrichment. In this regard, the present invention provides a method of human cancer treatment comprising: a) providing a human cancer patient, having one or more growing tumors; b) obtaining Vβ-expressing T cells from said patient that are sensitized to said growing tumor; c) culturing said T cells in a first culture media, said media comprising one or more superantigens so as to specifically stimulate a subset of Vβ-expressing T cells; d) culturing said T cells in a

second culture media, said media comprising human interleukin 2 so as to cause cell proliferation, thereby increasing the number of cells in said culture; and e) re-introducing at least a portion of said T cells into said patient so as to induce an *in vivo* therapeutic, tumoricidal reaction. In one embodiment, the method further comprises the step of administering human interleukin 2 to said patient *in vivo* after re-introducing said cells in step (e).

For culturing, the superantigen may comprise the enterotoxin SEB at concentrations above approximately 0.010 μg/ml. Preferably, the first culture media contains SEB at a concentration of approximately 2 μg/ml or greater and the second culture media contains human interleukin 2 at concentrations above 2 international units per milliliter.

DESCRIPTION OF THE FIGURES

Figure 1 schematically shows the therapeutic approach of the present invention.

Figures 2A, 2B, and 2C show a comparison of the primary sequences of the staphylococcal enterotoxins and their relatives.

DESCRIPTION OF THE INVENTION

The invention generally relates to the treatment of cancer, and more specifically, the treatment of solid tumors, including their metastases, without radiation, surgery or standard chemotherapeutic agents. In one embodiment, the invention involves a method wherein host cells are removed and stimulated outside the body, i.e., *ex vivo*, with stimulating antigens (see Figure 1). These stimulated cells are later reintroduced into the same host to mediate anticancer effects. When administered to subjects having tumors, the stimulated cells induce a tumoricidal reaction resulting in tumor regression.

It should be understood that the term, "tumoricidal reaction," as used herein, means that the tumor cells are killed, and is not meant to be limited to any particular method by which tumor cells are killed. For example, it may be that the tumor cells are killed directly (e.g., cell-cell interaction) or indirectly (e.g., release of cytokines like interferon) by the reinfused, stimulated cells. On the other hand, the stimulated cells, while not secreting cytokines themselves, may cause changes in paracrine growth signals.

With respect to the latter, it is known that metastatic cells receive and process negative paracrine growth signals, e.g., from molecules in the transforming growth factor-β family of cytokines. In conjunction with positive growth factors, the negative growth factors could determine metastatic cell growth at particular sites.

In one embodiment, the stimulating antigens are selected from among the staphylococcal enterotoxins. The staphylococcal enterotoxins and toxic shock syndrome toxin, have extraordinary properties as T cell antigens. Like other antigens, T cell stimulation by these toxins is believed to be dependent upon presentation by Major Histocompatability Complex (MHC) molecules. In contrast to conventional antigens, however, they apparently do not require presentation by a "self" MHC molecule; allogeneic antigen-presenting cells are equally effective. It is thought that the essential requirement is that cells presenting the toxins express MHC class II molecules, as these molecules specifically bind the toxins.

The staphylococcal toxins are believed not to be "processed" within antigen-presenting cells to oligopeptides that are displayed to T cells within the class II antigen-binding groove. Instead, it is postulated that the intact protein binds outside the groove and interacts directly with

T cell receptors for antigen. Most importantly, there is evidence that the staphylococcal toxins bind to a site on the Vβ segment of the T cell receptor heterodimer that is distinct from the complex site for binding of self MHC and foreign peptide antigen. Because the toxins do not bind to a site constituted by the full array of Vβ, Dβ, Jβ, Vα, and Jα gene products, the frequency of T cells responding to these molecules exceeds that of conventional peptide antigens by several orders of magnitude. Hence their name, "superantigens".

It is not intended that the invention be limited by the origin or nature of the host cells. Preferably, they are hematopoietic cells, such as immune cells (e.g., tumor infiltrating lymphocytes) or cells capable of developing into immune cells. While they may be isolated from a variety of sources, such as bone marrow (e.g., from femurs by aspiration), spleen or peripheral blood (e.g., collected with heparin and separated by Ficoll/hypaque gradient), as well as from the tumor (e.g., tumor-infiltrating lymphocytes). It is preferred that they are obtained from the lymph nodes. While they may be obtained from normal, disease-free donors, it is also preferred that they be obtained from tumor-bearing hosts.

Tumor-Draining Lymph Nodes

It has been known that tumor draining lymph nodes contain T cells specifically sensitized to the growing tumor, although such cells are insufficient to mediate an antitumor response. These cells, termed "pre-effector" cells, can differentiate into functional immune cells upon further *in vitro* stimulation. Several culture techniques have been developed for successful generation of antitumor effector cells from tumor draining lymph nodes. S. Shu *et al.*, J. Immun., 139:295-304 (1987). B. Ward *et al.*,

J. Immun., 141:1047-1053 (1988). T. Chou *et al.*, J. Immun., 141:1775-1781 (1988). Initially, irradiated tumor cells were used to drive the maturation of draining lymph node cells, and, more recently, anti-CD3 monoclonal antibody and IL-2 were used. H. Yoshizawa *et al.*, J. Immun., 147:729-737 (1991). However, the results reveal less than complete killing. While not limited by an understanding of the mechanism, this may be due to polyclonal stimulation with the particular stimulating agents used, i.e., generation of a significant proportion of immune cells with irrelevant specificity.

Superantigens As Stimulating Agents

The approach of the present invention is to use more effective stimulating agents. Again, while not limited by an understanding of the mechanism, it is believed that so-called "superantigens" are capable of selectively activating subsets of T cells responsible for mediating the desired immune response.

Among the best studied superantigens are enterotoxins produced by *Staphylococcus aureus*. These superantigens are single chain proteins with molecular weights ranging from 22,000 to 38,000, and more particularly between 24,000 and 30,000. They are heat stable and resistant to trypsin digestion (the general properties of the enterotoxins are given in Table 1A and 1B). According to one aspect of the present invention, enterotoxins isolated from media which are supporting the growth of various *Staphylococcus aureus* organisms are used.

The enterotoxins of *Staphylococcus aureus* form a group of serologically distinct extracellular proteins, designated A, B, C_1, C_2, C_3, D, E and F. These proteins are recognized as the causative agents of Staphylococcal food poisoning.

Enterotoxin F appears to be important in the pathogenesis of the Staphylococcal toxic shock syndrome.

It is not intended that the present invention be limited by the origin or nature of the particular enterotoxin. Indeed, synthetic polypeptides with substantial structural homology and with statistically significant sequence homology and similarity to Staphylococcal enterotoxins and Streptococcal pyrogenic exotoxins, including alignment of cysteine residues and similar hydropathy profiles, may also be effective stimulants *ex vivo* to induce a tumoricidal reaction when the stimulated cells are reinfused. In addition to enterotoxins, such peptides might be derived from, but are not limited to sequences in additional superantigens such as minor lymphocyte stimulating loci, mycoplasma and mycobacterial, *Yersinia* and *Streptococcal* Protein M antigens, heat shock proteins, stress peptides, and mammary tumor viruses.

The protein sequences and immunological cross-reactivity of the enterotoxins reveal that they can be divided into two related groups. The Staphylococcal enterotoxins A, E and D (SEA, SEE and SED) constitute one group, and Staphylococcal enterotoxins B and C (SEB, SEC) and Streptococcal pyrogenic exotoxin A (SPEA) make up the second group. Amino acid sequences show that SEA and SEE are almost identical and that SEB, SEC and SPEA share regions of similar sequence (amino acid sequence similarities and congruences are given in Tables 2-4). SED is moderately related to both groups although it is more similar to the SEA group. There is a striking amino acid similarity among enterotoxins A, B, C, D and E in the region immediately downstream from cysteine located at residue 106 in SEA. A second region at residue 147 also shows a highly conserved sequence.

TABLE 1A
Some Properties Of The Enterotoxins

	Enterotoxin			
	A[a]	B[b]	C_1[c]	C_2[d]
Emetic dose (ED_{50}) (µg/monkey)	5	5	5	5-10
Nitrogen content (%)	16.5	16.1	16.2	16.0
Sedimentation coefficient ($S_{20,w}$) (S)	3.04	2.78	3.00	2.90
Diffusion coefficient ($D_{20,w}$) (x $10^{-7}cm^2sec^{-1}$)	7.94	8.22	8.10	8.10
Reduced viscosity (ml/g)	4.07	3.81	3.4	3.7
Molecular weight	34,700	30,000	34,100	34,000
Partial specific volume	0.726	0.726	0.728	0.725
Isoelectric point	6.8	8.6	8.6	7.0
Maximum absorption (mµ)	277	277	277	277
Extinction ($E^{1\%}_{1cm}$)	14.3	14.4	12.1	12.1

[a] F.S. Thadhani *et al.*, Biochem., 5:3281 (1966).
[b] M.S. Bergdoll *et al.*, J. Bacteriol., 90:1481 (1965).
[c] C.R. Borja and M.S. Bergdoll, Biochem., 6:1467 (1967).
[d] R.M. Avena and M.S. Bergdoll, Biochem. 6:1474 (1967).

TABLE 1B
Physicochemical Properties Of Staphylococcal Enterotoxins[e]

	Enterotoxin					
Property	A[a]	B[b]	C_1[c]	C_2[d]	D[e]	E[f]
Emetic dose for monkey (µg)	5	5	5	5-10	-	-
Sedimentation coefficient ($S_{20,w}$)	3.03	2.89	3.0	2.9	-	2.6
Molecular weight	27,800	28,366[g]	26,000	34,100	27,300	29,600
Isoelectric point	7.26	8.6	8.6	7.0	7.4	7.0
C-terminal residue	Serine	Lysine	Glycine	Glycine	Lysine	Threonine
N-terminal residue	Alanine	Glutamic acid	Glutamic acid	Glutamic acid	Serine	-

[a] E.J. Schantz *et al.*, Biochem., 11:360 (1972).
[b] E.J. Schantz *et al.*, Biochem. 4:1011 (1965).
[c] C.R. Borja and M.S. Bergdoll, Biochem., 6:1467 (1967).
[d] R.M. Avena and M.S. Bergdoll, Biochem. 6:1474 (1967).
[e] P.C. Chang and M.S. Bergdoll, Biochem., 18:1937 (1979).
[f] C.R. Borja *et al.*, J. Biol. Chem., 247:2456 (1972).
[g] Data Section in *Atlas Protein Sequence Structure 5:D227*, (M. Dayhoff, ed.), National Biomedical Research Foundation, Washington D.C. (1972) (determined from the amino acid sequence of I.Y. Huang and M.S. Bergdoll, J. Biol. Chem., 245:3493 (1970)).
Modified from M.S. Bergdoll *et al.* in *Recent Advances in Staphylococcal Research*, (W.W. Yotis, ed.), Ann. N.Y. Acad. Sci., 236:307-316.

These regions are contained on the peptide fragment of SEC, and are known to contain the active sites for emesis and diarrhea. The mitogenic region resides in the C terminal tryptic fragment of SEC, implying that other regions of sequence similarity exist.

Comparison of the primary sequences of the staphylococcal enterotoxins and their relatives is shown in Figures 2A, 2B, and 2C. The complete primary amino acid sequences of the staphylococcal enterotoxins and related proteins are shown aligned, with the exception of the sequences of the exfoliating toxins, which are shown aligned with each other, but not with the remaining toxins. The exfoliating toxins have properties related to those of the others.

TABLE 2*
Sequence Similarities Among The Pyrogenic Toxins And Enterotoxins

Toxin	Sequence	
	106 119	147 163
SEA	CMYGGVTLHDNNRL	KKNVTVQELDLQARRYL
SEB	CMYGGVTEHHGNOL	KKKVTAQELDYLTRHYL
SEC1	CMYGGITKHEGNHF	KKSVTAQELDIKARNFL
SED	CTYGGVTPHEGNKL	KKNVTVQELDAQARRYL
SEE	CMYGGVTLHDNNRL	KKEVTVQELDLQARHYL
SPEA	CIYGGVTNHEGNHL	KKMVTAQELDYKVRKYL
Consensus	CMYGGVTLHEGNHL	KKNVTAQELD$^{L}_{Y}$QAR$^{R}_{H}$YL
TSST-1	IHFQISGVTNTEKL	KKQLAISTLDFEIRHQL

* J.J. Iandolo, Ann. Rev. Microbiol., 43:375 (1989).

TABLE 3
Amino Acid Composition Of The Enterotoxins (g/100g Protein)

	Enterotoxin				
Amino Acid	A*	B†	C_1‡	C_2‡	E§
Lysine	11.26	14.85	14.43	13.99	10.83
Histidine	3.16	2.34	2.91	2.87	3.04
Arginine	4.02	2.69	1.71	1.75	4.50
Aspartic acid	15.53	18.13	17.85	18.38	15.10
Threonine	5.96	4.50	5.31	5.80	6.36
Serine	2.99	4.05	4.58	4.81	4.72
Glutamic acid	12.36	9.45	8.95	8.93	12.15
Proline	1.35	2.11	2.16	2.23	1.93
Glycine	2.96	1.78	2.99	2.90	4.10
Alanine	1.94	1.32	1.85	1.61	2.38
Half-cysteine	0.66	0.68	0.79	0.74	0.81
Valine	4.93	5.66	6.50	5.87	4.36
Methionine	0.96	3.52	3.20	3.60	0.45
Isoleucine	4.11	3.53	4.09	4.02	4.30
Leucine	9.78	6.86	6.54	6.13	10.08
Tyrosine	10.63	11.50	9.80	10.27	9.79
Phenylalanine	4.31	6.23	5.35	5.25	4.47
Tryptophan	1.46	0.95	0.99	0.84	1.51
Amide NH_3	1.80	1.66	1.71	1.62	1.66
TOTAL	98.37	100.15	100.00	99.99	100.88

* Schantz *et al.*, 1972.
† M.S. Bergdoll *et al.*, Arch Biochem Biophys, 112:104 (1965).
‡ I.Y. Huang *et al.*, Biochem., 6:1480 (1967).
§ Borja *et al.*, 1972.
¶ M.S. Bergdoll *et al.*, Agric. Food Chem., 22:9 (1974).

TABLE 4†
Amino Acid Compositions Of TSST-1a And 1b[a]

Amino acid	Amino acid composition		
	TSST-1a residues per mole[b]	TSST-1b residues per mole[b]	Clone[b]
Aspartic acid	26	27	25
Threonine	21	20	19
Serine	20	20	21
Glutamic acid	20	20	17
Proline	10	8	10
Glycine	13	14	11
Alanine	4	5	3
Half-cysteine	0	0	0
Valine	5	5	5
Methionine	0	0	2
Isoleucine	15	15	17
Leucine	14	16	15
Tyrosine	10	8	9
Phenylalanine	7	7	7
Histidine	5	5	5
Lysine	23	24	21
Tryptophan	ND[d]	ND[d]	3
Arginine	4	5	4
	197	199	194

† D.A. Blomster-Hautamaa and P.M. Schlievert, Meth. Enzym., 165:37 (1988).

[a] Isolated from strain MN8, as compared to the inferred amino acid composition of the TSST-1 structural gene.

[b] Residues per mole values are based on a molecular weight of 22,000.

[c] Residues per mole inferred from the DNA sequence of the TSST-1 structural gene. Blomster-Hautamaa and colleagues.

[d] ND. Not determined.

The toxins shown in Figures 2A, 2B, and 2C are as follows: SEA to SEE, *Staphylococcus aureus* enterotoxins A to E; SPE A and C, *Streptococcus pyogenes* toxins A and C; TSST1, *Staphylococcus aureus* toxic shock - associated toxin; ETA and ETB, *Staphylococcus aureus* exfoliating toxins A and B. Single letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

It should be noted that the two Streptococcal toxins SPEA and C are about as similar to each of the Staphylococcal groups as they are to each other. Exfoliative toxins (ETA, ETB) are of similar size to SEB and SEA with similar modes of action. They share several points of sequence similarity to the Staphylococcal enterotoxins. Overall there are several stretches at which similarities are apparent throughout the total group comprised of Staphylococcal enterotoxins, Streptococcal pyrogenic exotoxins and Staphylococcal exfoliative toxins.

The recognition that the biologically active regions of the enterotoxins and SPEA were substantially structurally homologous enables one to predict synthetic polypeptide compounds which will exhibit similar tumoricidal effects. Table 6 illustrates the amino acid sequence homology of mature SPEA and *Staphylococcus aureus* enterotoxin B. The top sequence is the SPEA-derived amino acid sequence. The amino acid sequence of enterotoxin B is on the bottom. Sequences are numbered from the amino acid terminus, with amino acids represented by standard one character designations (see Table 5). Identities are indicated by : and gaps in the sequences introduced by the alignment algorithm are represented by dashed lines. [See L.P. Johnson *et al.*, Mol. Gen. Genet., 203:354-356 (1986).]

One common methodology for evaluating sequence homology, and more importantly statistically significant similarities, is to use a Monte Carlo analysis using an algorithm written by Lipman and Pearson to obtain a Z value. According to this analysis, a Z value greater than 6 indicates probable significance, and a Z value greater than 10 is considered to be statistically significant. W.R. Pearson and D.J. Lipman, Proc. Natl. Acad. Sci. (USA), 85:2444-2448 (1988); D.J. Lipman and W.R. Pearson, Science, 227:1435-1441 (1985).

In the present invention, synthetic polypeptides useful in tumoricidal therapy and in blocking or destroying autoreactive T and B lymphocyte populations are characterized by substantial structural homology to enterotoxin A, enterotoxin B and streptococcal pyrogenic exotoxins with statistically significant sequence homology and similarity (Z value of Lipman and Pearson algorithm in Monte Carlo analysis exceeding 6) to include alignment of cysteine residues and similar hydropathy profiles.

Toxicity of Superantigens

Previous approaches utilizing superantigens in cancer therapy have involved systemic exposure to these agents. Such early approaches include both plasma perfusion over a solid support matrix containing superantigens [D.S. Terman *et al.*, New Eng. J. Med., 305:1195 (1981)] as well as direct injection of superantigens into a tumor-bearing host. D.S. Terman, Patent Application Serial No. PCT/US91/00342 (1990); K.A. Newell *et al.*, Proc. Nat. Acad. Sci (USA), 88:1074 (1991).

It is believed that all enterotoxins are capable of inducing fever and shock when given systemically (e.g., intravenously). When administered in this manner, they are presumed to function by affecting emetic receptors in the

TABLE 5

Amino Acid	One-letter Symbol
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

TABLE 6

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                10        20                  30        40        50
 STR-PKPSQLQRSNLVKTFKIYIFFMRVTL-----VTHENVKSVDQLLSHDLIYNVS--
 :   :::  :  :   : :      : : :     :   ::::          ::::  :
ESQPDPKPDELHKSS--K-FTGLMENMKV-LYNNDHVSAINVKSINEFF--DLIYLYSIK
          10        20        30        40        50

            60        70        80        90
----GPNYDKLKTELKNQEMATLFKDKNVDIYGVEYYHLCYLC---------ENAERSAC
    : :::     : ::   :   ::: ::  :  ::  ::          ::   :  :
DTKLG-NYDNVRVEFKNKDLADKYKDKYVDVFGANYYQ-CYFSKKTNNIDSHENTKRKTC
      60        70        80        90        100       110

 100       110               120       130       140       150
LYGGVTNHEGNHLEIPKK----IVVKVSIDGIQSLSFDIEQIKNGNCSRIS-YTVRKYLT
 ::::: :   : :    :     : : :  ::   ::::    :          :  : ::
MYGGVTEHGNNQLD---KYYRSITVRVFEDGKNLLSFDVQTNKKKVTAEQLDYLTRHYLV
       120       130       140       150       160

      160       170       180       190       200
DNKQLYTNGPSKYETGYIKFIPKNKESFWFDFFPEPE--FTQSKYLMIYKDNETLDSNTS
 :: ::    : :::::::::  :  ::: :  : :   : :::::: :      ::
KNKKLYEFNNSPYETGYIKFIE-NENSFWYDMMPAPGNKFDQSKYLMMYNNDKMVDSKDV
170       180       190       200       210       220

         220
QIEVYLTTK
 ::::::::
KIEVYLTTKKK
 230
```

abdominal viscera which stimulate the emetic and diarrheal response. They are also believed to induce interferon, tumor necrosis factor, and interleukins 1 and 2. Unfortunately, the increased effectiveness of higher doses of systemically introduced superantigens is correlated with higher toxicity. In this regard, direct administration of increasingly effective, anti-cancer doses in animals has been followed by shock and death within 12-24 hours.

The present invention contemplates avoiding the undesirable effects, but nonetheless harnessing the valuable characteristics of superantigens. Preferably, there is no

significant systemic exposure to superantigens using the *ex vivo* stimulation approach of the present invention.

It should be noted that the *ex vivo* approach also allows for the presence of minor impurities in the preparation that would be unacceptable in preparations for direct administration. While these impurities might be toxic (or even lethal) *in vivo*, they can simply be washed away along with the superantigen itself following *ex vivo* culture.

In sum, the criteria for superantigens, and in particular, superantigen purity are: 1) mitogenic activity in a tritiated thymidine proliferation assay, 2) stimulation of interferon release, 3) Vβ cell reactivity, 4) amino acid profile (see above), 5) HPLC and PAGE (21-28,000 MW); 6) negative in the limulus amebocyte lysate (LAL) test for endotoxin; 7) negative in a hemolytic assay for the presence of alpha-hemolysin.

Ex Vivo Stimulation

As noted above, a number of cell types can be used. When cells from lymph nodes are used, all types of lymph nodes are contemplated (inguinal, mesenteric, superficial distal auxiliary, etc.). For *ex vivo* stimulation, they are removed aseptically and single cell suspensions are prepared by teasing under sterile conditions. Cell preparations then may be filtered (e.g., through a layer of nylon mesh), centrifuged and subjected to a gentle lysing procedure, if necessary.

Tumor-draining lymph node cells may be stimulated *in vitro* using a number of protocols. For example, a sufficiently large number of lymph node cells (i.e., a number adequate to show a tumoricidal reaction upon reinfusion) are exposed to superantigens (e.g., SEA, SEB, etc.) and diluted in synthetic culture media (e.g., RPMI

1640 with typical supplements) for the appropriate period of time (e.g., two days). Any number of standard culture techniques can be employed (e.g., 24-well plates in an incubator at 37°C in a 5% CO_2 atmosphere).

Following the incubation, the stimulated cells are harvested and washed with synthetic media containing no superantigens. At this point, the cells may be cultured further with other agents if desired (e.g., IL-2). In any event, the cells are counted to determine the degree of proliferation and resuspended in appropriate media for therapy.

The stimulated cells may be reintroduced to the host by a number of approaches. Preferably, they are injected intravenously. Optionally, the host may be treated with agents to promote the *in vivo* function and survival of the stimulated cells (e.g., IL-2).

Of course, the stimulated cells may be reintroduced in a variety of pharmaceutical formulations. These may contain such normally employed additives as binders, fillers, carriers, preservatives, stabilizing agents, emulsifiers, and buffers. Suitable diluents and excipients are, for example, water, saline, and dextrose.

Alternate Embodiments

Tumor resensitized lymphocytes may become anergized in the course of tumor growth *in vivo* and become refractory to activation or expansion by the superantigens with T cell Vβ specificity. Various cytokines may partially reverse T memory cell anergy, namely, IL-2, IL-4, or IL-1 plus IL-6. These cytokines may promote T cell proliferation and may represent an essential "second signal" typically provided by antigen presenting cells. Hence, responsiveness of tumor sensitized lymphocytes may be restored by co-culturing with

various cytokines and mitogens such as anti-CD3 antibody or conconavalin A.

While the preferred embodiment involves culturing *ex vivo*, other approaches are also contemplated. In one embodiment, the present invention contemplates transfecting with superantigen genes into tumor cells to provide powerful augmenting signals to T cell stimulation. In another embodiment, dual transfection with superantigens and molecules such as B7 is contemplated. Moreover, various cytokines and antibodies which are known to enhance T cell proliferation and secretion such as interleukin 1, interleukin 2, interleukin 4, interleukin 6, anti-CD3 or anti-CD2 may be employed simultaneously or sequentially with enterotoxins *in vivo* or *in vitro* to augment antitumor effects of the enterotoxins.

Substances which increase the number of anitgen-presenting cells, as well as substances which induce up-regulation of class II molecules on antigen-presenting cells or T cells, such as Y interferon, ICAM molecules and the like, used *in vitro* or *in vivo* could create additional binding sites for superantigen presentation to the T lymphocyte population and augment T lymphocyte proliferative and secretory function as well as anti-tumor effects.

Finally, various superantigens may be employed sequentially to up-regulate the activity of one another. For example, SEA, which is known to be a powerful cytokine inducer, may be used *in vitro* or *in vivo* to up-regulate class II molecules before the use of SEB or SEC, which are potent T cell stimulants. The up-regulated class II binding sites created by SEA would be occupied by SEB, providing significantly increased antigenic presentation to the T cell Vβ repertoire.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μM (micromolar); mM (millimolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μg (micrograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); °C (degrees Centigrade); mAb (monoclonal antibody); MW (molecular weight); U (units); d(days).

EXAMPLE 1

Production And Isolation Of Enterotoxins

This example describes two purification approaches for Enterotoxins A and C_2.

Approach 1: A 10 ml culture of *Staphylococcus aureus* 11N-165 (SEA), *Staphylococcus aureus* 361 (Source: Dr. John Iandolo, Kansas State University, Manhattan, KS) (SEC_2) is grown overnight at 37°C. The removal of enterotoxin from the supernatant is carried out using QAE-Sephadex. The toxin is then eluted batchwise from the ion exchanger and recovered by filtration on a sintered glass funnel. The eluates are concentrated by ultrafiltration. The toxin is then passed through a Sephadex-G-100 column. Two peaks absorbing at 280 mm are eluted, with the latter containing the enterotoxin. The eluted toxin is concentrated and rerun on Sephadex-G-100. The overall recovery is about 30% for SEC_2 and 40 to 50% for SEA. Both toxins appear homogeneous by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Approach 2: *Staphlococcus aureus* Strain FRI-722 is grown in a 3% enzyme-hydrolyzed casein and 7% yeast extract at pH 6.6 at a temperature of 35-37°C. The mixture is gently agitated for 16-20 hours. The culture is filtered through a 0.2 micron filter and the filtrate pH is adjusted to 5.6. The filtrate is diluted 1:5 to 1:10 with deionized water, incubated with a cation exchange resin and stirred for 1 h. The resin is collected and the bound protein is eluted with high ionic strength buffer. The eluate is concentrated and dialyzed then reincubated with a second cation exchange resin. The SEA is eluted with a low ionic strength to high ionic strength buffer gradient. The fraction containing SEA is concentrated, dialyzed and loaded onto a gel filtration system. The fraction containing SEA is concentrated and dialyzed against PBS pH 7.2. The final solution is filter-sterilized and frozen. Total protein is determined spectrophotometrically at 280/260 nm. A 5 µg/ml solution is tested in gel diffusion against a known antisera to SEA and 1 µg/ml is tested in PAGE and endotoxin in the Sigma-E-Toxate LAL assay.

EXAMPLE 2

Production And Isolation Of Enterotoxins

This example describes a purification approach for Enterotoxins A and C_1 and D.

This approach utilizes fast protein liquid chromatography (FPLC) and high resolution chromatofocusing Mono P column. Enterotoxins in media are concentrated and passed over a Sephadex-G-75 column. The toxin containing fractions are pooled. For C_1 and D, the supernatants are passed over an AmberLite-CG-50 column, as described for SED, and the active fractions pooled. All three toxins are then placed in buffer for chromatofocusing and then separated

using the MONO P column FPLC system. Since all of the toxins have isoelectric points in the range of 7 to 9, the polybuffer PBE-96 is used for elution. The purity of SEA, SEC_1 and SED is estimated to be 98, 95 and 80%, respectively. SEA elutes as two peaks at pH 8.8 and 8.6. SEC_1 also elutes as two peaks at pH 8.3 and 7.9, and SED elutes as three peaks at pH 8.6, 8.3 and 8.0.

Enterotoxins may also be produced in mutant strains of *Staphylococcus aureus* by expression of an enterotoxin producing gene in another bacteria or cell. Genetic material which appears to be in the chromosomal plasmid, or phage portion of the bacteria may be used for gene insertion procedures. Complete molecules or fragments with amino acid sequence homology to the parent enterotoxin may be produced with this technology. (Reviewed in Iandolo, J.J., Annu. Rev. Microbiol., 43:375 (1989). Moreover, mutagenic agents such as N-Nitroso compounds are capable of augmenting significantly the production of enterotoxins by some strains of Staphylococcus.

EXAMPLE 3

Production And Isolation Of Enterotoxins

This example describes a purification approach for Alpha Toxin.

Staphylococcus aureus Wood 46 strain (Source: Dr. Sidney Harshman, Vanderbilt University, Nashville, TN) is used and cultured in yeast extract dialysate medium. With the glass-pore bead method undialyzed yeast may be used together with casein, glucose, thiamine and nicotinic acid. The organism is incubated in medium for 24h at 37°C.

The culture supernatant is applied to a glass- pore bead column and adjusted to pH 6.8. A column of 5 x 20 cm is used for 3 liter batches and flow rates adjusted to 10-20

ml/min. The column is washed with 0.01M $KHPO_4$ pH 6.8 and then the alpha toxin is eluted with 1.0M $KHPO_4$ pH 7.5. Fractions are tested for the presence of alpha hemolysin by a rapid hemolytic assay using rabbit erythrocytes as substrate.

EXAMPLE 4

Production And Isolation Of Enterotoxins

This example describes a purification approach for Streptococcal Pyrogenic Exotoxin (SPE).

Streptococcus NY-5 strain (Source: ATCC 12351) has been the most widely used for toxin production and studies. A list of various strains to produce toxins A, B, and C has been published. The Kalbach S84 type 3 strain (Source: Dr. Joseph E. Alouf, Institute Pasteur-Unite Associee, Paris, France) is cultured and the supernatant is concentrated and stirred in calcium phosphate gel. Fraction S_1 is precipitated with 80% saturated ammonium sulfate. The redissolved pellet is dialyzed and designated Fraction S_2. This fraction is precipitated with 50-80% ammonium sulfate, resuspended in phosphate buffered saline (Fraction S_3), and gel filtered on a Bio-Gel P-100 column. The fraction corresponding to the volume eluted between 160 and 240 ml is collected and concentrated by ultrafiltration to about 20 ml in an Amicon PM10 Membrane (Fraction S_4). Fraction S4 is then submitted to preparative isoelectric focusing (IEF) performed with a 100 ml column. The material which focuses at around pH 4.8 in a narrow peak is collected and dialyzed in an Amicon cell using PBS to eliminate ampholines and sucrose. The Fraction (S_5) constitutes purified pyrogenic exotoxin. Another electrophoretic form of SPE with a pI of 4.2 is often separated simultaneously with that of pI 4.8.

Both forms show total cross reactivity against immune sera raised by rabbit immunization with fraction S_3.

The Fraction S_5 shows a single band by SDS-PAGE corresponding to a molecular weight of 28K. Bioassays for determination of activity include erythematosus skin test in rabbits or guinea pigs lymphocyte blast transformation. The toxin may also be detected by enzyme-linked immunoabsorbant assay (ELISA) or hemagglutination inhibition.

EXAMPLE 5

Production And Isolation Of Enterotoxins

This example describes a general purification approach for native enterotoxins.

Current methods for purification of all of the enterotoxins utilize ion exchange materials such as CG-50, carboxymethyl-cellulose and the Sephadexes (gel filtration). The preparation of the SEB used for these studies is as follows.

Staphylococcus aureus strain I10-275 is cultured in NZ-Amine A media supplemented with 10 g/liter of yeast extract for 18-20 hours in room air at 37°C. The flask is agitated at 300 RPM. The initial pH of the culture is 6.8 and the postincubation pH 8.0. The culture is filtered through a DC-10 Amicon filter (pore size 0.1 micron). The final filtrate is adjusted to pH 5.6. The filtrate is tested for the presence of SEB in radial immunodiffusion using known antisera to SEB. Eighteen to 20 liters of culture supernatant fluid are diluted with deionized, distilled H_2O (1:5 to 1:10) and the pH adjusted to 5.6, CG-50 resin (Malinkrodt) (800 ml), preequilibrated to pH 5.6 in 0.03 M phosphate buffer, pH 6.2 (PB) is added and the mixture stirred for one hour. The resin is allowed to settle and the supernatant fluid is decanted. The resin is placed in a

column and the toxin is eluted with 0.5 M PB, 0.5 M NaCl pH 6.2. The concentrated, dialyzed toxin is placed in a column (5 cm x 75 cm) of CM-sepharose (pretreated with 0.005 M PB pH 5.6). The column is washed with the same buffer and the enterotoxin eluted by treating the column stepwise with PB 0.03 M pH 6.0, 0.045 M pH 6.25, 0.06 M pH 6.5 and 0.12 M pH 7.2. The fractions containing the enterotoxin are combined, concentrated with polyethylene glycol (200 ml wet volume of packed resin), and dialyzed against 0.5 M NaCl 0.05 M PB pH 7.2. The concentrated enterotoxin solution (5 ml) is placed in a column of Sephacryl S-200 (pretreated with 0.5 M NaCl, 0.05 M PB, pH 7.2). The column is eluted with the same buffer. The fractions containing the enterotoxin are combined and dialyzed against 0.01 M PB, 0.15 M NaCl pH 7.2. The enterotoxin B concentration is approximately 1 mg/ml. The solution is filter sterilized, frozen and lyophilized. Samples are stored in lyophilized form at 4°C. The final enterotoxin fraction is a white powder which, when dissolved in normal saline, is a clear colorless solution. Samples containing 5 and 10 µg/ml are tested in a double diffusion immunoprecipitation assay using known standards of SEB and mono-specific antisera. A single precipitation line is noted which showed a line of identity with known SEB. Using a tritiated thymidine mitogenic assay with human and murine immunocytes, SEB showed significant mitogenic activity comparable to that of SEA. SEB was found to be devoid of contaminating alpha hemolysin assessed in a rabbit erythrocyte hemolytic assay.

PAGE gel analysis of SEB showed a predominant single band at 28,000 m.w. High performance liquid chromatography (HPLC) profiles were obtained on a MAC PLUS controlling a Rainin Rabbit HPLC with a Hewlett Packard 1040 A Diode array detector and a Vyadac Protein and Peptide C18 column. The profile for purified enterotoxin B was a sharp peak without

significant shoulder. There was minimal trace contamination. Amino acid analysis was carried out with a Bechman 6300 amino acid analyzer and displayed residues consistent with known SEB standards. The sterility of the preparations was demonstrated by negative cultures in thioglycolate medium and soybean-casein digest. Protein determinations were carried out by a spectrophotometric method.

The sterility of the preparation was demonstrated by negative cultures using (a) fluid thioglycollate medium and (b) soybean-casein digest. A sample containing 1 mg/ml of SEB was tested for endotoxin contamination using Sigma E-toxate LAL assay. The final product was found to be free of endotoxin with a standard sensitivity of 0.1 ug endotoxin/mg SEB.

Toxicity testing was carried out in two Hartley strain guinea pigs weighing less than 450 grams, and two female C57 black mice (Simonson Laboratories, Watsonville, CA), weighing less than 22 grams. Each animal was observed for 7 days with no significant change in condition or weight after intraperitoneal injection of 0.5 ml of 26 µg/kg enterotoxin B.

SEA, SEC, SED, SEE, TSST-1 and Streptococcal pyrogenic exotoxin in the studies were prepared by the previously described methods. The identity, purity and sterility of these preparations were tested in a fashion similar to that for SEB.

EXAMPLE 6

Isolation Of Host Cells: Lymph Nodes

As noted previously, the invention involves, in one embodiment, a method wherein host cells are removed and stimulated outside the body, i.e., *ex vivo*, with stimulating antigens. These cells may be isolated from a variety of sources. In this example, they are obtained from the lymph nodes.

Inguinal, mesenteric, or superficial distal axillary lymph nodes are removed aseptically. Single cell suspensions are prepared by teasing (e.g., with 20-gauge needles) followed by pressing mechanically with the blunt end of a 10-ml plastic syringe plunger in buffer under sterile conditions. The cell preparations were filtered through a layer of No. 100 nylon mesh (Nytex; TETKO Inc., Elmsford, NY), centrifuged and washed. Red cells, if evident, are lysed by treatment with ammonium chloride-potassium lysing buffer (8.29 g NH_4Cl, 1.0 g $KHCO_3$, and 0.0372 g EDTA/liter, pH 7.4). The cells were washed twice with buffer and resuspended for stimulation.

EXAMPLE 7

Isolation Of Host Cells: Spleen Cells

In this example, the host cells are obtained from the human spleen. Either a left subcostal incision or midline incision may be used for resection. The spleen is mobilized initially by dividing the ligamentous attachments, which are usually avascular. The short gastric vessels then are doubly ligated and transected. This permits ultimate dissection of the splenic hilus with individual ligation and division of the splenic artery and vein.

The sequence of technical maneuvers necessary to remove the spleen varies somewhat, depending on the surgeon's election to approach the splenic hilum either anteriorly or posteriorly. The anterior approach is somewhat slower.

Anterior Method. On entering the abdomen, the stomach should be thoroughly emptied by suction through a nasogastric tube already in place, if this maneuver has not been accomplished preoperatively. An opening is made in the gastrosplenic omentum in an avascular area, and by retracting the stomach upward and anteriorly through this opening the upper part of the pancreas can be visualized. The tortuous splenic artery can be seen along its upper margin; it is, at the option of the surgeon, ligated.

The next step in the procedure is division of the lower two-thirds of the gastrosplenic omentum. This is accomplished by dividing the vascular omentum between clamps and ligating the cut ends subsequently. The gastrosplenic omentum is frequently infiltrated with a considerable amount of adipose tissue and tends to slip away from clamps, especially if traction is applied to the instruments. The upper portion of this omentum also contains the vasa brevia and large venous tributaries joining the left gastroepiploic vein. To avoid hemorrhage from these sources, suture ligation rather than simple ligatures should be utilized in this area. Access to the upper portion of the gastrosplenic omentum is difficult with the spleen *in situ*, and for this reason it is best divided with the later stage after mobilization of the splenic hilum.

Following division of the splenic vasculature, the splenorenal, the splenocolic, and the splenophrenic ligaments are divided. All except the last mentioned are generally avascular and pose no particular technical problems in division. The remnants of the splenophrenic

ligament left behind may have to be underrun with running chromic catgut suture for hemostasis. The spleen is displaced from the abdomen and delivered through the incision. The only remaining attachments still in place is the upper third of the gastrosplenic ligament which is now carefully divided between ligatures, completing the splenectomy procedure.

Posterior Method. The posterior approach of removing the spleen is much more expeditious than the anterior approach, but blood loss is usually more substantial than in the anterior approach. After entering the abdomen the surgeon makes an incision in the avascular splenorenal ligament and then inserts three fingers behind the hilum of the spleen which is easily mobilized by blind dissection. Hemorrhage from the splenic hilum during this process can be avoided by placing the incision on the splenorenal ligament closer to the kidney and away from the spleen. By rapidly dividing the splenophrenic and the splenocolic ligaments, it is now possible to deliver the spleen through the incision. Any hemorrhage from the splenic hilum or from the ruptured spleen itself is very easily controlled at this point by manual compression of the splenic hilum or placement of a noncrushing clamp, taking care not to injure the tail of the pancreas. The gastrosplenic ligament and the presplenic fold when present can now be divided and suture ligated in a deliberate manner.

Cell Suspensions. Spleen cells are mechanically dissociated by using the blunt end of a 10-ml plastic syringe in buffer. The cell suspension was passed through a single layer of 100-gauge nylon mesh (Nitex; Lawshe Industrial Co., Bethesda, MD) and centrifuged, and the RBC lysed by resuspension of the cell pellet in ammonium

chloride/potassium lysing buffer, (8.29 g of NH_4Cl, 1.0 g $KHCO_3$ and 0.0372 g of EDTA/L pH 7.4; Media Production Section, National Institutes of Health, Bethesda, MD). The cells were again filtered through nylon mesh, washed two times, and resuspended in culture medium (see below).

EXAMPLE 8

Isolation Of Host Cells: Infiltrating Cells

In this example, the host cells are obtained from tumor infiltrating lymphocytes. Lymphocytes infiltrating tumors are obtained using standard techniques. Solid tumors (freshly resected or cryopreserved) are dispersed into single cell suspensions by overnight enzymatic digestion [e.g., stirring overnight at room temperature in RPMI 1640 medium containing 0.01% hyaluronidase type V, 0.002% DNAse type I, 0.1% collagenase type IV (Sigman, St. Louis), and antibiotics]. Tumor suspensions are then passed over Ficoll-Hypaque gradients (Lymphocyte Separation Medium, Organon Teknika Corp., Durham, NC). The gradient interfaces contain viable tumor cells and mononuclear cells are washed, adjusted to a total cell concentration of 2.5 to 5.0 x 10^5 cells/ml and cultured in complete medium. Complete medium comprises RPMI 1640 with 10% heat-inactivated type-compatible human serum, penicillin 50 IU/ml and streptomycin 50 µg/ml (Biofluids, Rockville, MD), gentamicin 50 µg/ml (GIBCO Laboratories, Chagrin Falls, OH), amphotericin 250 ng/ml (Funglzone, Squibb, Flow Laboratories, McLean, VA), HEPES buffer 10 mM (Biofluids), and L-glutamine 2 mM (MA Bioproducts, Walkersville, MD). Conditioned medium from 3- to 4-day autologous or allogeneic lymphokine-activated killer (LAK) cell cultures (see below) can be added at a final concentration of 20% (v/v). Recombinany IL-2 (kindly

supplied by the Cetus Corporation, Emeryville, CA) can be added at a final concentration of 1000 µ/ml.

Cultures are maintained at 37°C in a 5% CO_2-humidified atmosphere. A variety of tissue culture vessels can be employed, including 24-well plates (Costar, Cambridge, MA). 175 cm^2 flasks (Falcon; Becton Dickinson, Oxnard, CA), 850 cm^2 roller bottles (Corning Glass Works, Corning, NY), and 750 cm^2 gas-permeable culture bags (Fenwal Laboratories, Division of Travenol Laboratories, Deerfield, IL). Cultures should be fed weekly by harvesting, pelletting and resuspending cells at 2.5 x 10^6 cells/ml in fresh medium. Over an initial period (e.g., 2 to 3 weeks) of culture, the lymphocytes will selectively proliferate, while the remaining tumor cells will typically disappear completely.

To make LAK cell cultures, peripheral blood lymphocytes (PBL) are obtained from patients or normal donors. After passage over Ficoll-Hypaque gradients, cells are cultured at a concentration of 1 x 10^6/ml in RPMI 1640 medium with 2% human serum, antibiotics, glutamine, and HEPES buffer. Recombinant IL-2 is added at 1000 µ/ml. Cultures are maintained for 3 to 7 days in a humidified 5% CO_2 atmosphere at 37°C.

EXAMPLE 9

Ex Vivo Stimulation

This example describes an approach to stimulate host cells *in vitro* with superantigens for reinfusion. Tumor-draining lymph node (LN) cells are obtained as described in Example 7 and stimulated *in vitro* in a procedure with an optional second step.

Step One. For stimulation, 4×10^6 LN cells, in 2 ml of culture medium containing SEA or SEB, are incubated in a well of 24-well plates at 37°C in a 5% CO_2 atmosphere for 2 days. The culture media comprises RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 0.1 mM nonessential amino acids, 1 µM sodium pyruvate, 2 mM freshly prepared L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 50 µg/ml gentamicin, 0.5 µg/ml fungizone (all from GIBCO, Grand Island, NY) and 5×10^{-5} M 2-ME (Sigma). The cells were harvested and washed.

Step Two. The initially stimulated cells are further cultured at 3×10^5/well in 2 ml of culture media with Human recombinant IL-2 (available from Chiron Corp., Emeryville, CA; specific activity of 6 to 8 $\times 10^6$ U/mg protein; units equivalent to 2-3 International U). After 3 days incubation in IL-2, the cells can be collected, washed, counted to determine the degree of proliferation, and resuspended in media suitable for intravenous (i.v.) administration (e.g., physiological buffered saline solutions).

EXAMPLE 10

Immunotherapy

As noted previously, the present invention involves stimulating cells *ex vivo*, allowing them to differentiate into tumor specific immune effector cells. The cells are then reintroduced into the same host to mediate anticancer therapeutic effects.

In this example, 8 to 12 week old female C57BL/6J (B6) mice (Jackson Laboratory, Bar Harbor, ME) are injected i.v. with approximately 3×10^5 MCA 205 tumor cells (i.e., methylcholanthrene-induced tumors of B6 origin provided by Dr. James Yang, Surgery Branch, National Cancer Institute,

Bethesda, MD) suspended in 1 ml of media to initiate pulmonary metastases. These tumors can be routinely passed *in vivo* in syngeneic mice and used within the third to seventh transplantation generation.

On day 3, cells obtained from the mice as in Example 6 are stimulated *ex vivo* as in Example 9. Specifically, LN cells draining progressively growing MCA 205 fibrosarcoma for 12 d are stimulated with graded concentrations of SEA or SEB for 2 d followed by culture in 4 U/ml of IL-2 for 3 d.

The antitumor efficacy of superantigen stimulated cells is assessed by reinfusion. Mice may also be treated with exogenous IL-2 to promote the growth of transferred cells (i.p, with 15,000 U IL-2 in 0.5 ml buffered saline twice daily for 4 consecutive days to promote the *in vivo* function and survival of the stimulated cells). On day 20 or 21, all mice can be randomized, sacrificed, and metastatic tumor nodules on the surface of the lungs enumerated.

To identify Vβ phenotypes of cells in the tumor-draining LN before and after SEA and SEB stimulation, cells can be stained with a collection of anti-Vβ mAb. A preferential stimulation of particular Vβ T cell subsets by different microbial superantigenic toxins would suggest the possibility of antigenic specificity of the responding T cells.

From the above, it should be clear that the present invention provides a method for the treatment of cancer, and, more specifically, for the treatment of solid tumors, including their metastases, without radiation, surgery or standard chemotherapeutic agents. The *ex vivo* stimulation method has decided advantages over direct intravenous injection of superantigens. Most importantly, success is achieved with minimal host toxicity.

CLAIMS

1. A method for inducing a tumoricidal reaction *in vivo* comprising contacting cells with superantigens *ex vivo* and infusing them into a tumor-bearing host.

2. The method of Claim 1 whereby said cells are hematopoietic cells.

3. The method of Claim 2 whereby said cells are lymph node cells.

4. The method of Claim 3 whereby said lymph node cells are from said tumor-bearing host.

5. The method of Claim 1 whereby said superantigens comprise enterotoxins of *Staphylococcus aureus*.

6. The method of Claim 1 whereby said superantigens comprise synthetic polypeptides with substantial structural homology and statistically significant sequence homology to natural superantigens.

7. A method of human cancer treatment comprising:

a) providing a human cancer patient;

b) obtaining hematopoietic cells from said patient;

c) contacting said cells *ex vivo* with one or more superantigens to generate stimulated cells; and

d) re-introducing said stimulated cells into said patient so as to induce an *in vivo* therapeutic, tumoricidal reaction.

8. The method of Claim 7 wherein said hematopoietic cells are spleen cells.

9. The method of Claim 7 wherein said hematopoietic cells are lymph node cells.

10. The method of Claim 7 wherein said hematopoietic cells are peripheral blood lymphocytes.

11. The method of Claim 7 wherein said superantigens comprise enterotoxins of *Staphylococcus aureus*.

12. The method of Claim 11 wherein said hematopoietic cells are cultured in culture media containing said enterotoxins.

13. The method of Claim 12 wherein said cultured cells are washed prior to re-introducing said stimulated cells into said patient so as to essentially avoid introducing enterotoxins *in vivo*.

14. A reagent for treating cancer, comprising T cells sensitized to a growing tumor and stimulated with superantigens.

15. The reagent of Claim 14, wherein said T cells are from tumor draining lymph nodes.

16. The reagent of Claim 14, wherein said T cells are suspended in media suitable for intravenous administration to a human cancer patient

17. The reagent of Claim 16, wherein said media comprises a physiological buffered saline solution.

FIGURE 1

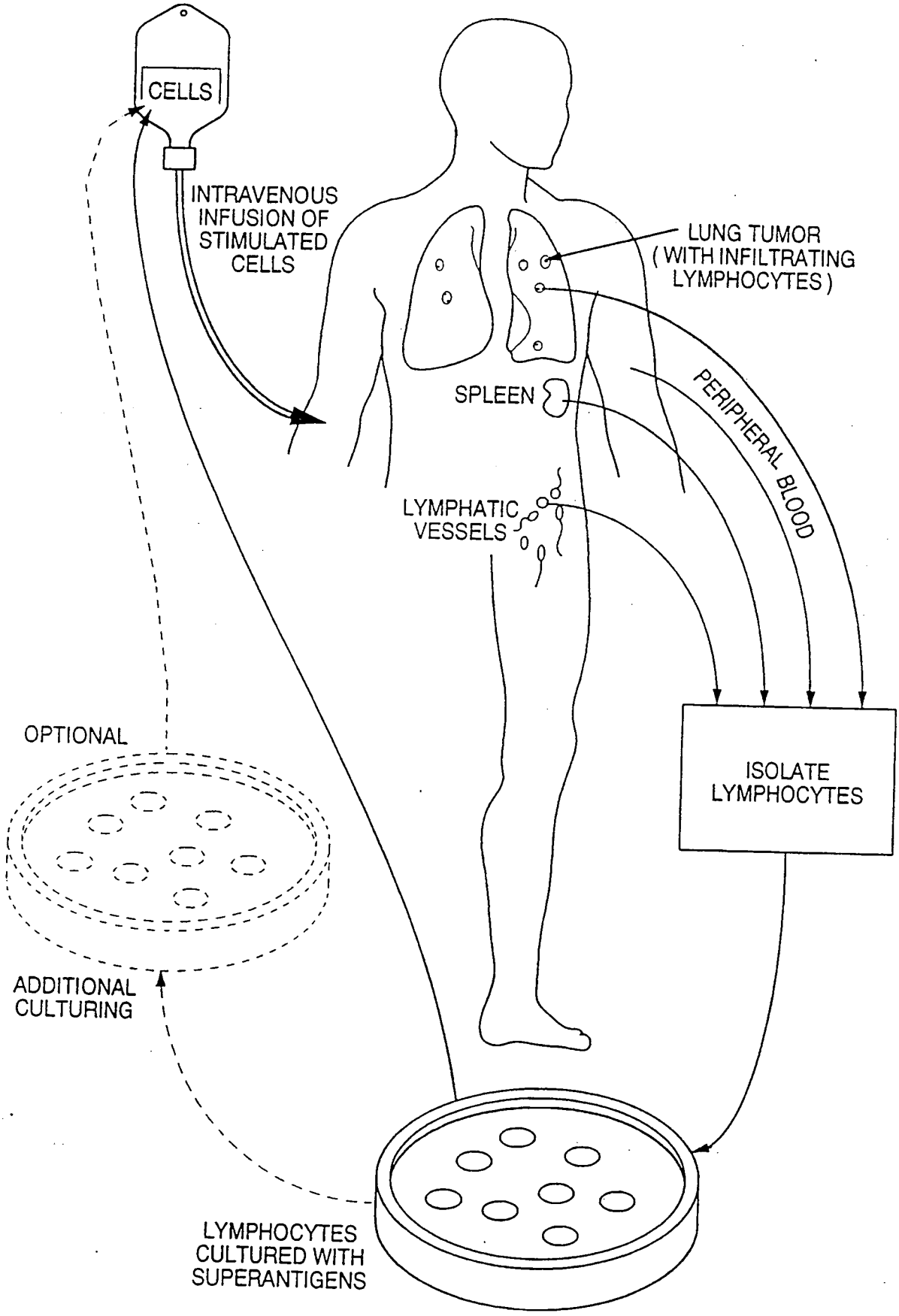
CELLS
INTRAVENOUS INFUSION OF STIMULATED CELLS
LUNG TUMOR (WITH INFILTRATING LYMPHOCYTES)
SPLEEN
PERIPHERAL BLOOD
LYMPHATIC VESSELS
OPTIONAL
ISOLATE LYMPHOCYTES
ADDITIONAL CULTURING
LYMPHOCYTES CULTURED WITH SUPERANTIGENS

FIGURE 2A

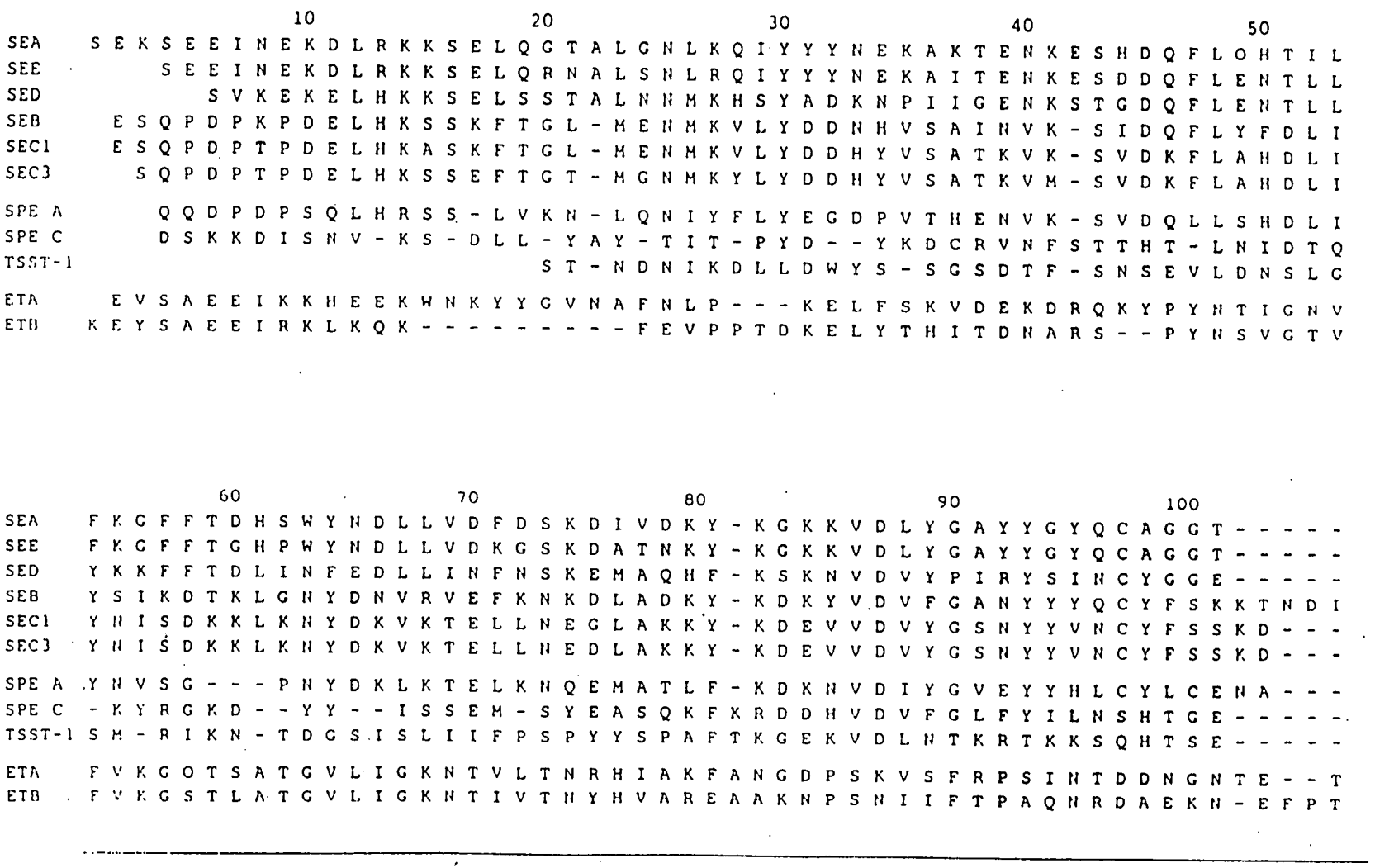

FIGURE 2B

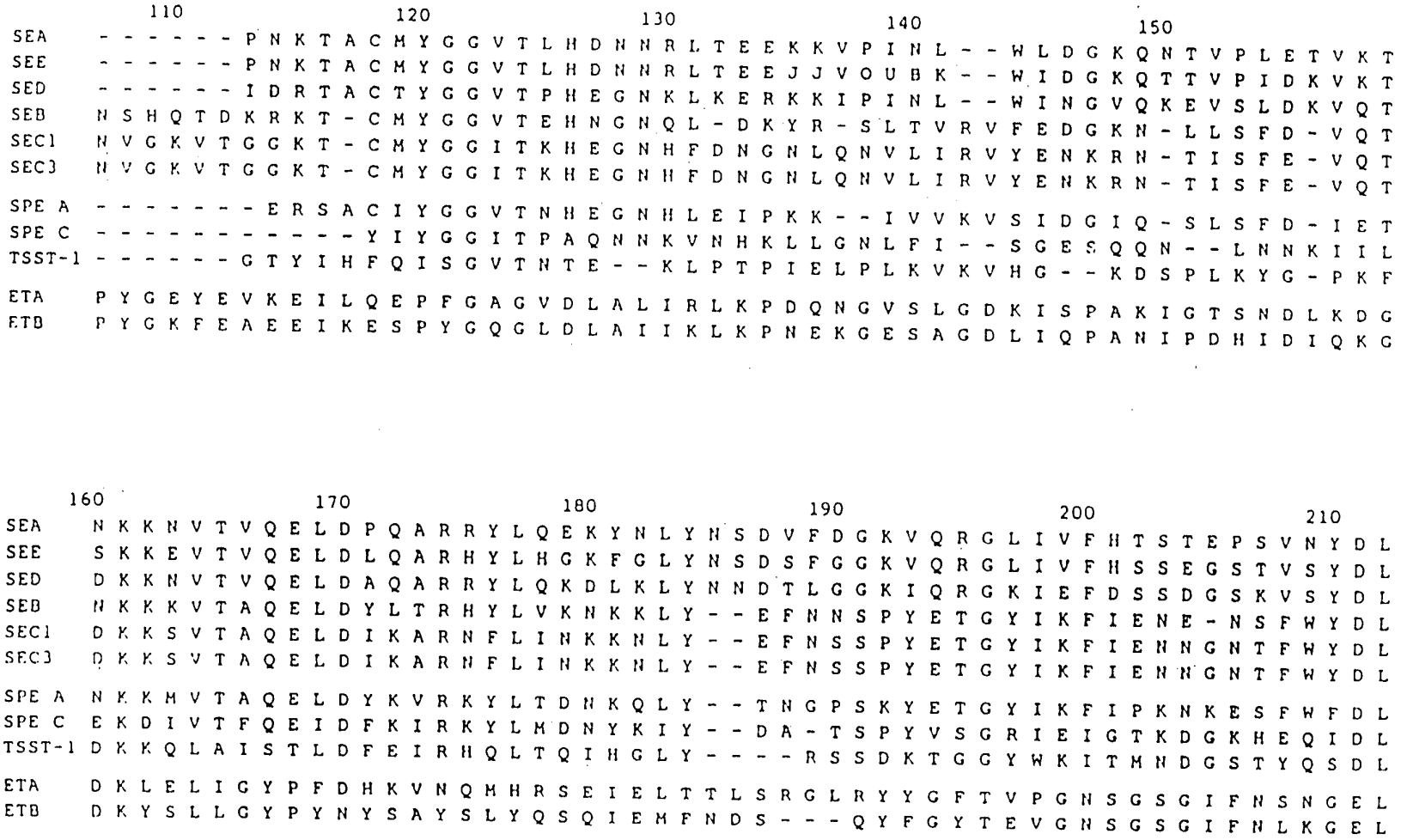

FIGURE 2C

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                220       230       240       250       260
SEA   FGAQGQYSNT--LLRIYRDNKTINSE-NMHIDIYLYTS*
SEE   FDAQGQYPDT--LLRIYRDNKTINSE-NMHIDIYLYTT*
SED   FDVKGDFPE--KQLRIYSDNKTLSTE-HLHIDIYLYEK*
SEB   HPAPGDKFDQSKYLMNYNDNKMVDSK-DVKIEVYLTYKKK*
SEC1  HPAPGDKFDQSKYLMHYNDNKTVDSK-SVKIEVHLTTKNG*
SEC3  HPAPGDKFDQSKYLMHYNDNKTVDSK-SVKIEVHLTTKNG*
SPE A FPEP-EFT-QSKYLMIYKDNETLDSN-TSQIEVYLTTK*
SPE C FDSPNEGT-RSDIFAKYKDNRIINMKNFSHFDIYL-EK*
TSST-1 SKF-EYN-TEKPPINIDEIKTIEAEIN*
ETA   VGIHSS----------------KVSHLDREHQINYGVGIGNYV-KRIINEKNE*
ETB   IGIHSGKGGQHNLPIGVFFHRKISSLYSVDNT-FGDTLGNDLKKRAKLDK*
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02339

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) : A61K 37/00, 39/00; A61M 37/00
US CL : 424/88; 514/2; 604/4, 5, 6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/88; 514/2; 604/4, 5, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Volume 88, No. 3, issued February 1991, Newell et al, "*In Vivo* T-cell Activation by Staphylococcal Enetrotoxin B prevents Outgrowth of a Malignant Tumor", pages 1074-1078, see at least the Abstract.	1-17
Y	Cambridge Science Abstracts, issued 1993, Scheglovita et al.," EffectStaphylococcal Enterotoxin A-sensitized spleen Cells on Metastasizing of Mouse Lewis Carcinoma", abstract no. 1477534, Eksp. Onkol., 11(2), pages 54-56, see entire abstract.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
02 JUNE 1994

Date of mailing of the international search report
14 JUN 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02339

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Infection and Immunity, Volume 57, No. 7, issued July 1989, Garcia-Penarrubia et al, "Selective prolfertion of Natural Killer Cells among Monocyte-Depleted peripheral Blood Mononuclear Cells as a Result of Stimulation with Staphylococcal Enterotoxin B", pages 2057-2065, see at least the Abstract.	14-17
Y	The New England Journal of Medicine, Volume 313, No. 23, issued 05 December 1985, Rosenberg et al, "Observations on the Systemic Administration of Autologous Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 to Patients with Metastatic Cancer", pages 1485-1492, see at least the Abstract.	14-17